TRANSLATION NO. 84

DATE: Sept 1968

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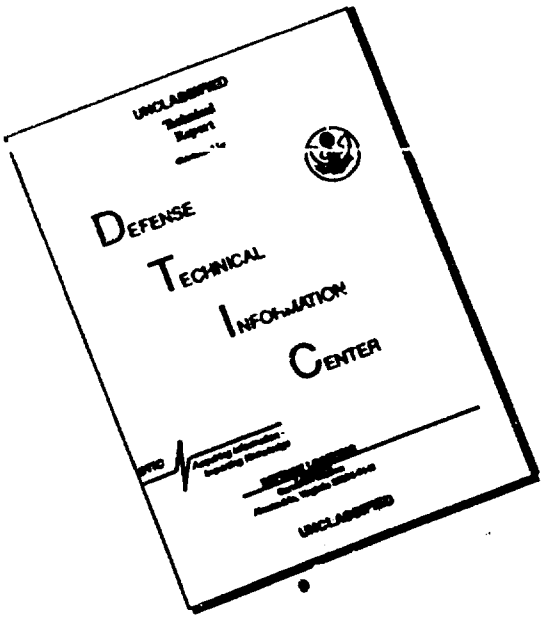

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Problems of Virology, USSR, No. 2, 1956, Pages 43-47

Experimental Evaluation of New Laboratory Methods for the Study of Virus of Poliomyelitis With the Aid of Tissue Cultures, by V. I. Zhevandrova (Institute of Study of Poliomyelitis, AMS, USSR, Moscow)

During the past 5 years great achievements in the development of methods of laboratory study of the virus of poliomyelitis have been attained. Thanks to the application of tissue cultures it was possible to isolate the virus from infectious material and identify it in cultures with specific serums, determine the level of antibodies in the serums, obtain great quantities of the virus for the production of diagnostic antigens and prophylactic vaccines, study the biochemical activity of the virus and the action of various chemical substances on it in order to obtain new medicinal properties.

Irregardless of these achievements, to the present time there is still not a sufficiently simple method of cultivating the poliomyelitis virus in vitro, although the number of suggested methods almost equal the number of researchers working in this field.

This report contains the results of our tests on the cultivation of the virus in cultures of surviving tissues in Karrel vessels and in cultures of actively growing tissues in revolving test tubes according to Enders.

Besides this, we reviewed the possibility of cultivating the types II and IV virus of poliomyelitis in Erlich's acetic carcinoma in white mice according to the method suggested by A. I. Ivanenko (Strain types I and III are pathogenic only for monkeys, strain type II for monkeys, cotton rats and grown white mice, strain LE (type IV) is pathogenic for monkeys, cotton rats and sucklings of white mice).

The original material for the infection of the culture was the brain and spinal cord substances of infected animals in a 10% suspension or a developed 20% suspension of excrement of patients. Into each tissue culture was put 0.2 ml of virus containing material. The fecal suspension was centrifuged 1 hour at 4000 rpm before introduction and also 1000-2000 units of penicillin and 250-500 units of streptomycin were added to 1 ml of the settling fluid. Presence of the virus in the cultures was determined by infection of susceptible animals and also by the presence of specific degenerative variations in the cells of the tissue.

The reaction of neutralization of the virus in the cultures was done as follows: typospecific immune serums in dilutions 1:10 were mixed with equal volumes of dilutions of 1:10 or whole cultural virus fluid. The resulting mixture was held for 30 minutes to 1 hour at room temperature and was added to test tubes with cultures of fibroblasts at 0.2 ml. Serums without virus or viruses without serum were added to the control cultures. Also, several unworked tissue controls were left.

Preparation of Cultures of the Surviving Tissues. Brain or skin and muscle tissues of a 3-5 month human embryo were aseptically cut with scissors into pieces with a diameter of 1-2 mm, were washed of tissue fluid and blood with a salt solution and 2 drops of the tissue pulp were put into the Karrel vessels. The suspension of virus was poured directly on the tissue and held 10-15 minutes at room temperature after which 2 ml of a nutritive fluid was added to the vessels (3 parts balanced salt solution of Hanks and 1 part of beef or sheep serum which had been run through the Zeitsa disk; penicillin 100-150 units to 1 ml and streptomycin at 1000 Y or more per 1 ml). The fluid phase of the medium was

removed after 3-5 days and replaced with a nutritive fluid of the said consistence. On the 11-16th day the tissue was removed, ground, suspended in a nutritive fluid and mixed with fresh tissue on that same day or after being held on ice at 15-20°C for some time.

Preparation of Cultures of Actively Growing Tissues. Embryo tissue of man was obtained from 10-14 week old embryos in the form of fresh sterile scrapings of the womb surface. Nutritive mediums containing embryonal extract and normal (ordinarily placental) serum of human blood were used. Good results were obtained during application of the medium No. 27(7) which consisted of amniotic fluid of blood (45%), Hanks salt solution (25%), placental or donor blood of a grown individual (20%), bovine embryonal extract (10%) and penicillin and streptomycin respectively at 50 and 100 units per 1 ml of medium.

When the fibroblasts had grown around the pieces of tissue to an area of 1 cm in diameter the cultures were transferred from the medium No. 27 to medium No. 47(7) which consisted of amniotic blood fluid (70%), filtered beef serum (15%) and Hanks salt solution (15%). In this medium the cultures free themselves from the antibodies to the virus of poliomyelitis which remain from the No. 27 medium.

The order of preparation of the cultures in the revolving test tubes was as follows: first 1-2 drops of chicken heparinized plasma was put into the tubes and it was dispersed in a thin layer over the inside surface of the bottom 2/3 of the tubes, then with a pipette we added several (5-15) pieces of tissue. The pieces of tissue were so dispersed that they were at a more or less equal distance from each other, and then they added 1-2 drops of chicken embryonal extract for the coagulation of the plasma. To insure an even formation of the plasma clot

the tubes were revolved approximately 1-2 minutes until the full coagulation was obtained. They allowed the forming clots to take form while holding the tubes 20-30 minutes in a horizontal position, after which each tube received 1-2 ml of the nutritive fluid with pH 7.4-7.6 (this was determined by the variation of the brightness of the indicator of the phenol red during passage of mixtures of 50% carbon dioxide and air through the medium); then the tubes were covered tightly with rubber stoppers and placed in an almost horizontal position in special drums in a thermostatic room at 35°C.

The apparatuses with the rotating drums for the cultivation of the tissue in the tubes were prepared in the workshop of the Institute of Virology, D. I. Ivanovski, AMS, USSR according to designs of K. P. Chumakova, M. I. Voroshilova and A. G. Kirillova. The apparatus consists of a double reduction gear which is powered by an electric phonograph motor with a constant number of revolutions, to which are attached two drums with 150 or 700 tubes each.

The axis of the drums are angled horizontally 5° so that the stoppers of the tubes would not be moistened by the nutritive fluid. The drums rotate around the axis constantly at 10-12 revolutions per hour.

The nutritive fluid in the cultures was changed every 2-5 days. Small cells grow up around the pieces of tissue by the 4th day forming a multicomplex syncytium which consisted of drawn out, spindle-shaped and star-like cells with bright oval nuclei and a small number of rounded roaming cells of various sizes interspersed among them. Along with the splendid growing of the small cells, on the 4-5th day, there appeared sections of intervals around the tissue pieces as a result of the opening of the clot of the plasma to the growing cells - so-called lysis of the plasma which is the greatest complication during this method of

cultivation. A particularly significant lysis was noted in cultures with growing epithelial elements. The increasing rarification of the clot of the plasma caused a slipping down of the tissue pieces from the walls of the tubes, compression of the zone of growth around the fields of lysis which hindered the growth of the fibroblasts. Therefore, we prophylactically conducted additional feeding of the cultures until the appearance of an expressed lysis on the 4-5th day, that is, additional layers of plasma in order to renew the plasma clot. To do this the nutritive fluid was removed from the tubes and plasma and embryonal extract were mixed and evenly layered on the surface of the culture. 20-30 minutes after the formation of the new clot the additionally fed cultures received the nutritive fluid. In those cultures with growing epithelial elements the renewal of the clot of the plasma from kidney, tongue and skin was conducted every 4-5 days which significantly complicated work with them.

The cultures in the revolving tubes were usually ready for infection with virus on the 6-7th day. At this time, having preliminarily poured off the nutritive fluid, the cultures with actively multiplying cells received material containing virus. The contact of the virus and cells lasted 10-20 minutes after which the nutritive fluid was added to the tubes.

Test Results

It was possible to obtain a distinct multiplication of the virus of poliomyelitis type I (Strain KRF) and II (Strain Lansing) in cultures on Karrel vessels with brain and skin-muscular tissue surviving embryonal tissue of man; there was a much better multiplication of several strains of virus types I, II and III in cultures with growing skin-muscular tissue in revolving tubes. One series of the strain Lansing was sustained by us in a culture on surviving brain

tissue for more than 2½ years. During this time the virus went through 17 passages on the Karrel vessels and then was transferred to a culture in the revolving tubes. The original dose of virus in the process of passages was diluted 10^{18} times, if we consider it according to the changes of the tissue, and 10^{51} times, considering the changes of the fluid medium, which illustrates the unconditional multiplication of the virus. In similar series 6-12 passages of the virus type I and II were conducted.

Our tests indicate that in cultures with surviving tissues of embryo of man (brain, skin and muscle) it is possible to obtain a regular multiplication of the virus of poliomyelitis and duratively sustain the virus outside an organism, but in cultures of this type the virus accumulates in small quantities. Its presence in the culture can be judged only by the results of infection of susceptible animals and data of histological studies of the cuts of tissue which complicates identification of the virus and makes it more durative.

A distinctly more advantageous method is the growing of the virus of poliomyelitis in revolving tubes with actively growing tissue. Having been cultivated in these cultures the virus causes characteristic degenerative variations of the cells which are seen easily during inspection of the tubes with cultures under a microscope. The degenerative variations of the growing cells are first indicated in the rounding, swelling and accumulation of lipoid and other products of change in them. According to the accumulation of the virus these variations increase and the cells are fully disrupted forming granular detritus.

The degenerative variations of the cells caused by all the strains of virus of poliomyelitis tested by us, were morphologically identical and were expressed in necrosis of young cells. The variations of the cells were direct indicators of the presence of an active virus of poliomyelitis in the cultures; in the

presence of a homological typical immune serum they did not multiply.

The ability of this virus to cause necrotic variations of the young multiplying cells and be neutralized by typical immune serum was utilized by us for the isolation of new strains of virus from excrement of children ailing with poliomyelitis (strain Alekseev, Osadchaya) and determined the type origin of 9 strains of virus of poliomyelitis. Three of them proved to be strains of type I, two - type II and one strain (Eremeev) as yet has not been classified because it does not correspond to any type of serum we have. This strain requires additional study.

In cultures of embryonal tissue of cotton rats and white mice (strain Lansing and No. 113 type II and strain AE type IV) and also in acetic carcinoma of white mice (strain Lansing and Ovchinnikov type II and strain B type IV) it was not possible to detect multiplication of the poliomyelitis virus.

Thus, our observations fully conform with literature data on the possibility of a quick isolation and typification of strains of virus of poliomyelitis with the aid of cultures in revolving tubes.

The cultures of fibroblasts at this time are already widely used in our labs for examinations of virus-vectorability.

CONCLUSIONS

1. The multiplication of poliomyelitis types I, II and III viruses in cultures with surviving and growing tissues (brain, skin and muscle) of human embryo has been established.

2. The virus of poliomyelitis, during multiplication in tissue cultures, caused a characteristic necrosis of the cells. The cytopathogenic action of the virus of poliomyelitis was easily established during inspection under a

microscope of the cultures with young growing cells. After the neutralization of the virus with a typospecific immune serum this effect disappeared.

3. The possibility of typing the virus with a reaction of neutralization in the tissue culture was confirmed and determination of the type of strains of virus, isolated from excrement of ailing children in cultures in revolving tubes, was also accomplished.

4. In tests of cultivation of the poliomyelitis virus type II and IV in cultures of embryonal tissues of cotton rats and white mice and also in the acetic carcinoma of white mice it was impossible to establish multiplication of the virus.

5. The obtained results fully confirm the great advantages of the methods of cultivating virus of poliomyelitis on growing tissues in revolving tubes, allowing for the interchange in numerous studies of the tests of animals.

Material and Methods

Virus. 12 strains of the poliomyelitis virus were studied(see Table)

Utilized strains of poliomyelitis virus

Name of strain	Local of isolation	Date	Source	Mode of isolation	Virus type	Isolated by
KRF-1	Moscow	1950	Excrement of patients	Monkey	I	M K Voroshilova, M P Chumakov
ZK-UP	Alma-Ata	1954	" "	"	I	M K Voroshilova
Lug-mozg	" "	1954	Brain	Monkey	I	M k Voroshilova
Lug-mindal	" "	1954	Tonsils	"	I	M K Voroshilova
Lansing	Lansing, USA	1937	Brain	"	II	Armstrong(USA)
113-L	Moscow	1949	Excrement	Cotton rats	II	E N Levkovich
Alekseev	"	1953	"	Sucklings of cotton rats	II	V I Zhevandrova
Ovchinnikov	"	1953	"	Same	II	Same
Alekseev	"	1953	"	Tissue culture	II	Same
Osadchaya	Alma-Ata	1954	"	Same	II	Same
Osadchaya	" "	1954	"	Sucklings of cotton rats	II	Same
Leon	USA	1937	Brain	Monkey	III	Committee on Virus classification
AB	Karaganda	1952	Excrement	"	IV	M K Voroshilova
Eremeeva	Alma-Ata	1954	Excrement	Cotton rats	--	V I Zhevandrova